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Endothelial Nitric Oxide Synthase Mediates Cutaneous Vasodilation During Local Heating And Is Attenuated In Middle-Aged Human Skin

By: Rebecca S. Bruning, Lakshmi Santhanam, Anna E. Stanhewicz, **Caroline J. Smith**, Dan E. Berkowitz, W. Larry Kenney, and Lacy A. Holowatz

Abstract

Local skin heating is used to assess microvascular function in clinical populations because NO is required for full expression of the response; however, controversy exists as to the precise NO synthase (NOS) isoform producing NO. Human aging is associated with attenuated cutaneous vasodilation but little is known about the middle aged, an age cohort used for comparison with clinical populations. We hypothesized that endothelial NOS (eNOS) is the primary isoform mediating NO production during local heating, and eNOS-dependent vasodilation would be reduced in middle-aged skin. Vasodilation was induced by local heating (42°C) and during acetylcholine dose-response (ACh-DR: 0.01, 0.1, 1.0, 5.0, 10.0, 50.0, 100.0 mmol/l) protocols. Four microdialysis fibers were placed in the skin of 24 men and women; age cohorts were 12 middle-aged (53 ± 1 yr) and 12 young (23 ± 1 yr). Sites served as control, nonselective NOS inhibited [NG-nitro-L-arginine methyl ester (L-NAME)], inducible NOS (iNOS) inhibited (1400W), and neuronal NOS (nNOS) inhibited (N ω -propyl-L-arginine). After full expression of the local heating response, L-NAME was perfused at all sites. Cutaneous vascular conductance was measured and normalized to maximum (%CVCmax: Nitropress). L-NAME reduced %CVCmax at baseline, all phases of the local heating response, and at all ACh concentrations compared with all other sites. iNOS inhibition reduced the initial peak (53 ± 2 vs. $60 \pm 2\%$ CVCmax; $P < 0.001$); however, there were no other differences between control, nNOS-, and iNOS-inhibited sites during the phases of local heating or ACh-DR. When age cohorts were compared, NO-dependent vasodilation during local heating (52 ± 6 vs. $68 \pm 4\%$ CVCmax; $P = 0.013$) and ACh perfusion (50 mmol/l: 83 ± 3 vs. $93 \pm 2\%$ CVCmax; 100 mmol/l: 83 ± 4 vs. $92 \pm 3\%$ CVCmax; both $P = 0.03$) were reduced in middle-aged skin. There were no differences in NOS isoform expression obtained from skin biopsy samples between groups (all $P > 0.05$). These data suggest that eNOS mediates the production of NO during local heating and that cutaneous vasodilation is attenuated in middle-aged skin.

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Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin

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Bruning RS, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL, Holowatz LA. Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin. *J Appl Physiol* 112: 2019–2026, 2012. First published April 12, 2012; doi:10.1152/jappphysiol.01354.2011.—Local skin heating is used to assess microvascular function in clinical populations because NO is required for full expression of the response; however, controversy exists as to the precise NO synthase (NOS) isoform producing NO. Human aging is associated with attenuated cutaneous vasodilation but little is known about the middle aged, an age cohort used for comparison with clinical populations. We hypothesized that endothelial NOS (eNOS) is the primary isoform mediating NO production during local heating, and eNOS-dependent vasodilation would be reduced in middle-aged skin. Vasodilation was induced by local heating (42°C) and during acetylcholine dose-response (ACh-DR: 0.01, 0.1, 1.0, 5.0, 10.0, 50.0, 100.0 mmol/l) protocols. Four microdialysis fibers were placed in the skin of 24 men and women; age cohorts were 12 middle-aged (53 ± 1 yr) and 12 young (23 ± 1 yr). Sites served as control, nonselective NOS inhibited [N^G -nitro-L-arginine methyl ester (L-NAME)], inducible NOS (iNOS) inhibited (1400W), and neuronal NOS (nNOS) inhibited (N^w -propyl-L-arginine). After full expression of the local heating response, L-NAME was perfused at all sites. Cutaneous vascular conductance was measured and normalized to maximum (%CVC_{max}; Nitropress). L-NAME reduced %CVC_{max} at baseline, all phases of the local heating response, and at all ACh concentrations compared with all other sites. iNOS inhibition reduced the initial peak (53 ± 2 vs. 60 ± 2 %CVC_{max}; $P < 0.001$); however, there were no other differences between control, nNOS-, and iNOS-inhibited sites during the phases of local heating or ACh-DR. When age cohorts were compared, NO-dependent vasodilation during local heating (52 ± 6 vs. 68 ± 4 %CVC_{max}; $P = 0.013$) and ACh perfusion (50 mmol/l: 83 ± 3 vs. 93 ± 2 %CVC_{max}; 100 mmol/l: 83 ± 4 vs. 92 ± 3 %CVC_{max}; both $P = 0.03$) were reduced in middle-aged skin. There were no differences in NOS isoform expression obtained from skin biopsy samples between groups (all $P > 0.05$). These data suggest that eNOS mediates the production of NO during local heating and that cutaneous vasodilation is attenuated in middle-aged skin.

inducible nitric oxide synthase; neuronal nitric oxide synthase; skin blood flow; vascular aging

THE CUTANEOUS MICROVASCULATURE provides an easily accessible, minimally invasive circulation to examine vascular function (1, 3, 11). Several methods are available to locally induce vasodilation in the cutaneous microvasculature. Direct local warming of the skin just below the pain threshold to 42°C

elicits near maximal vasodilation in young, healthy individuals because at this temperature the vascular smooth muscle is completely relaxed and gives a highly reproducible skin blood flow response. This protocol (17, 23, 24) has been increasingly utilized to examine microvascular dysfunction in clinical populations (10, 17, 25, 32). The skin blood flow response to local heating is biphasic, consisting of an initial sensory nerved-mediated rapid increase in skin blood flow, followed by a slower secondary rise to a plateau that is ~70% reliant on NO-dependent mechanisms (14, 17, 24). In addition to local heating, perfusion of the endothelium-dependent agonist acetylcholine (ACh) in a dose-dependent manner can be utilized to examine receptor-mediated endothelial function (21, 32).

All three NOS isoforms (endothelial, neuronal, and inducible) have been purported to contribute to the skin local heating response in different patient populations (18, 34). Significant controversy exists as to which is the primary isoform mediating the production of NO during the plateau phase of the skin local heating response. We have recently shown that an upregulation of the proinflammatory signaling mediator inducible nitric oxide synthase (iNOS) contributes to attenuated NO-dependent cutaneous vasodilation in essential hypertensive humans (32) potentially through S-nitrosylation of arginase 1, which competes for the NOS substrate L-arginine (30). Whether iNOS may contribute to the local heating response in nonpathological states is unclear.

Changes in both resistance vessel structure and decrements in NO-mediated dilation occur with aging and the development of cardiovascular disease (7, 29). Primary human aging, even in the absence of cardiovascular disease, is associated with attenuated skin blood flow responses including reduced plateau and relative NO-dependent cutaneous vasodilation during local heating and perfusion of endothelium-dependent receptor agonists (19, 26). However, little is known about these mechanisms along the aging continuum, specifically, in the middle-aged cohort often used for comparison with clinical disease populations.

Considering 1) the important role of NO in vascular health, 2) the increased utilization of local skin heating to assess microvascular function, 3) the conflicting evidence for both eNOS (18) and nNOS (34) involvement in NO synthesis during local heating, and 4) possible changes to these mechanisms with healthy middle aging, the primary aim of this study was to identify the predominant NOS isoform mediating cutaneous vasodilation during local heating. A secondary aim was to determine if there are any changes in these mechanisms with primary middle-aging. We hypothesized 1) that eNOS would be the predominant NOS isoform mediating cutaneous vaso-

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dilation during local heating, and 2) that eNOS-mediated vasodilation would be attenuated in healthy, middle-aged humans. In addition to functional *in vivo* studies, *in vitro* analysis of skin biopsy samples was also performed to examine NOS isoform expression and the downstream molecular vasodilatory target vasodilator-simulated phosphoprotein (pVASP) as an index of NOS activity.

METHODS

Subjects

All experimental procedures were preapproved by The Pennsylvania State University Institutional Review Board and met the guidelines set by the Declaration of Helsinki. Twelve middle-aged and twelve younger men and women voluntarily participated in the study after giving written and verbal informed consent. All subjects were screened prior to participating in the experiment to ensure they were nonsmokers, and free of dermatologic, metabolic, and cardiovascular diseases. In addition, all subjects' blood pressures were monitored for 24 h using an ambulatory blood pressure monitor (Ambulo 2400, Portland, OR) to ensure they were normotensive. Participants were normally active and were not taking any cardiovascular medications or supplements, oral contraceptives, or hormonal replacement therapy. All women were either postmenopausal or were tested in the low hormone phase of their menstrual cycles.

Instrumentation and Measurements

Subjects arrived in the room temperature (23°C) laboratory between 7:00 and 9:00 am dressed in comfortable clothing, typically exercise pants and a T-shirt for both protocols. Upon arrival subjects were instrumented with four intradermal microdialysis (MD) fibers (10 mm, 20-kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN) as previously described (32). Once inserted, the MD agents were perfused at a rate of 2.0 μ /min (Bee Hive control and Baby Bee microinfusion pumps; Bioanalytical Systems) for 60–90 min to ensure resolution of local hyperemia from needle insertion trauma.

In Vivo Analysis of Vascular Function

To obtain an index of skin blood flow, laser-Dopplers were (LDF; MoorLAB, Temperature Monitor SHO2, Moor Instruments, Devon, UK) placed in local heating units directly over the membrane portion of each MD fiber. The local heaters were set at 33°C to clamp local skin temperature during baseline measurements in the local heating protocol and throughout the ACh dose-dependent perfusion protocol. This localized temperature was used to ensure that the changes in percent of maximal cutaneous vascular conductance (%CVC_{max}) were due to the perfusion of ACh. Arterial blood pressure was measured every 5 min in the right arm throughout the experiment using a CardiCap blood pressure monitor and was verified with brachial auscultation. Mean arterial pressure (MAP) was calculated as the diastolic blood pressure plus one-third the pulse pressure. CVC was calculated as red blood cell flux divided by MAP.

Experimental protocols. Pharmacological agents were perfused during the 60- to 90-min resolution of the local hyperemic response from needle insertion trauma. After the local hyperemic response subsided, baseline recordings were measured for 20 min or until the hyperemic response had subsided. Each subject participated in two protocols (local heating and ACh perfusion in a dose-dependent manner) that took place on two different days and were separated by a minimum of 1 wk. During the protocols MD fiber sites were randomly assigned 1) lactated Ringer's solution to serve as control, 2) 20 mM *N*^G-nitro-L-arginine methyl ester (L-NAME; Tocris, Ellisville, MO) to nonselectively inhibit all NOS isoforms (NOS-I), 3) 0.1 mM *N*-[3-(aminomethyl)benzyl]acetamidine (1400W; Calbiochem,

San Diego, CA) to selectively inhibit iNOS (iNOS-I), and 4) 5 mM *N*^ω-propyl-L-arginine (NPLA; Tocris, Ellisville, MO) to selectively inhibit nNOS (nNOS-I). All inhibitors were dissolved in lactated Ringer's solution and were sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI). The efficacy of the isoform-specific antagonism and concentrations of the pharmacological agents used in this study have been demonstrated in other microdialysis studies (1400W K_i = 7 nM, NPLA K_i = 57 nM) (2, 4, 12, 22, 30, 34, 37). Furthermore, the efficacy of NPLA was also piloted with a whole body heating protocol in four young subjects to determine if the concentration used attenuated reflex vasodilation similar to those reported by Kellogg et al. (15).

Local heating. After 20 min of baseline measurements, the local heaters were increased at a rate of 0.5°C every 5 s to a temperature of 42°C. The sites were held at 42°C until a stable CVC plateau was reached (~30–40 min). After the stable plateau, while the local heater temperature was maintained at 42°C 20 mM L-NAME was perfused at a rate of 4 μ /min for 40–50 min at all sites to determine the reduction in CVC from inhibition of all NOS isoforms. This allowed for the within-site calculation of the percent reduction in CVC after NOS inhibition. The total local heating time was between 70 and 90 min.

ACh dose-response. After the baseline measurements ACh perfusion in a dose-dependent manner was performed to assess endothelial derived eNOS-mediated dilation (12, 20, 27). Just prior to the ACh perfusion, ACh doses (0.01, 0.1, 1, 5, 10, 50, and 100 mM) were diluted with the site-specific pharmacological agents. The doses were administered for 5 min in increasing concentrations (~40 min). This amount of time elicited a stable plateau of at least 3 min. At the end of each protocol, all sites were perfused with 28 mM sodium nitroprusside (SNP; Nitropress, Abbott Laboratories, Chicago, IL) at a rate of 4 μ /min and were locally heated to 43°C to ensure maximal CVC had been achieved.

In Vitro Analysis of Vascular Function

Ventral forearm skin samples were obtained from the opposite arm on a separate day from the *in vivo* functional assessment of vasoreactivity. The skin was anesthetized using 2% lidocaine without epinephrine. Using sterile technique two 3-mm-diameter skin samples were obtained. Samples were rinsed in lactated Ringer's and immediately frozen in liquid nitrogen and stored at –80°C until analysis.

Western blot analysis. After centrifugation of skin homogenates twice at 15,000 *g* at 4°C for 20 min, protein concentration was determined using a Bio-Rad DC protein assay. For Western blot analysis, 25 μ g (for eNOS and nNOS) and 50 μ g (for iNOS and pVASP) proteins were fractionated by SDS/PAGE and electrotransferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences). The membranes were blocked for 1 h at room temperature (5% nonfat dry milk, in Tris-buffered saline containing 0.1% Tween-20; TBST) and incubated with a primary antibody to eNOS (Santa Cruz Biotech; 1:1,000); nNOS (BD Bioscience, 1:1,000); iNOS (BD Bioscience, 1:1,000); and pVASP (1:1,000; Cell Signaling). Bound antibody was detected with horseradish peroxidase-conjugated IgG secondary antibody (1:1,000) (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence. Next, the pVASP blot was stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific) and reprobed with VASP antibody (Cell Signaling). GAPDH was used as loading control. Densitometry analyses were performed using ImageJ software (NIH).

Data acquisition and statistical analysis. Skin blood flow data were normalized as a percentage of maximal CVC (%CVC_{max}). Data were collected at 40 Hz, digitized, recorded, and stored in a personal computer until data analysis (Windaq, Dataq Instruments, Akron, OH). CVC data were averaged over a stable 5 min of baseline, local heating plateau, post-L-NAME plateau, and maximal vasodilation. The initial sensory-nerve peak was calculated as the highest average values (30-s intervals). The vasodilation due to NO at the plateau was

Table 1. *Subject characteristics*

	Young	Middle-Aged
Sex, M/W	7/5	3/9
Age, yr	23 ± 1	53 ± 1*
Height, cm	168 ± 3	174 ± 3
Weight, kg	81 ± 5	68 ± 3*
BMI, kg/m ²	27 ± 1	25 ± 1
Resting MAP, mmHg	87 ± 2	85 ± 2
Fasting glucose, mg/dl	89 ± 1	85 ± 2
Total cholesterol, mg/dl	148 ± 5	190 ± 5*
LDL, mg/dl	86 ± 7	108 ± 4*
HDL, mg/dl	44 ± 3	61 ± 5*
Cholesterol ratio, total/HDL	3.4 ± 0.3	3.2 ± 0.2

Values are means ± SE. M, men; W, women; BMI, body mass index; MAP, mean arterial pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. * $P < 0.05$: difference vs. young.

calculated from the difference between the plateau and the post-L-NAME plateau. CVC for each ACh-dose was averaged over the last 3 min of perfusion of each concentration and normalized to maximal vasodilation which was obtained from the most stable 5-min period where skin blood flow had peaked with the infusion of 28 mM SNP and with simultaneous locally heating to 43°C.

Student's unpaired t -tests were used to determine significant differences between age groups for physical characteristics and the reduction in NO-mediated dilation after perfusion of L-NAME during the local heating protocol. Data were initially analyzed using a three-way mixed model repeated-measures ANOVA (group × microdialysis treatment site × phase of the local heating response or ACh dose) (SAS, version 9.2, Cary, NC). When the groups were collapsed data were analyzed using a two-way mixed models repeated-measures ANOVA (microdialysis treatment site × phase of local heating response or ACh dose). A priori specific planned comparisons were performed when appropriate to determine where differences between groups and localized drug treatments existed. The level of significance was set at $\alpha = 0.05$ for main effects. All values are expressed as means ± SE.

RESULTS

Subject characteristics are presented in Table 1. A total of 24 subjects were tested. Age groups were well matched for height, body mass index (BMI), MAP, and cholesterol ratio (total cholesterol/high-density lipoprotein cholesterol). Absolute maximal CVC are presented in Table 2. There was no effect of age or localized microdialysis drug treatment on absolute maximal CVC (all $P > 0.05$).

During our initial pilot experiments to determine the efficacy of the nNOS inhibitor NPLA, relative NO-dependent vasodilation (assessed with L-NAME) was attenuated with pretreatment of 5 mM NPLA (control 28 ± 8 vs. NPLA $8 \pm 5\%CVC_{max}$).

Table 2. *Absolute maximal cutaneous vascular conductance*

Site	Young	Middle-Aged
Control	1.70 ± 0.22	1.87 ± 0.23
NPLA (nNOS inhibited)	1.94 ± 0.28	1.75 ± 0.18
1400W (iNOS inhibited)	1.92 ± 0.22	1.85 ± 0.23
L-NAME (all NOS inhibited)	1.86 ± 0.21	1.75 ± 0.20

Group means ± SE of absolute maximal cutaneous vascular conductance (flux/mmHg) for the 4 treatment sites in young and middle-aged groups. NPLA, *N*^ω-propyl-L-arginine; L-NAME, *N*^G-nitro-L-arginine methyl ester; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase.

Figure 1 shows the time course of the local heating response in a control site from a representative subject. All of the distinct phases of the response are labeled.

There was no interaction between age and NOS isoform-specific inhibitor during local heating or ACh dose-response; therefore, data for both age groups were combined to illustrate the differences due to specific NOS isoform inhibition. Figure 2 shows the mean data for the distinct phases of the local heating response in all drug treatment sites. Nonspecific NOS inhibition with L-NAME decreased baseline (Fig. 2A), the initial peak (Fig. 2B), and the plateau (Fig. 2C) compared with all other sites (all $P < 0.001$). iNOS inhibition reduced the initial sensory nerve-mediated peak (53 ± 2 vs. $60 \pm 2\%CVC_{max}$; Fig. 2B, $P < 0.001$), but there were no other differences between specific NOS-inhibited treatment sites. Figure 3 illustrates the age-group combined $\%CVC_{max}$ response for all drug treatment sites with increasing concentrations of the endothelium-receptor agonist ACh. L-NAME decreased $\%CVC_{max}$ across all concentrations of ACh (all $P < 0.001$). Compared with the control site there were no other differences with isoform-specific NOS inhibition.

Because there were no differences between sites with specific NOS isoform inhibition (NPLA and 1400W), data for the control site for the two age groups are presented in Figs. 4 and 5. Figure 4 shows the NO-dependent plateau and the post-L-NAME plateau of the local heating response. A quantification of total within-site NO-dependent vasodilation is illustrated with arrows and values provided. There were no differences in the plateau, but the middle-aged group had an augmented post-L-NAME plateau. Therefore, the within-site NO-dependent vasodilation was reduced in the middle-aged group (52 ± 6 vs. $68 \pm 4\%CVC_{max}$; $P = 0.013$). Figure 5 shows the $\%CVC_{max}$ response with increasing concentrations of ACh separated by age. The middle-aged group had attenuated ACh-induced vasodilation at the highest concentration (100 mmol/l: 83 ± 4 vs. $92 \pm 3\%CVC_{max}$; $P = 0.03$).

Figure 6 shows densitometric analysis and representative Western blots of nNOS, iNOS, eNOS, and pVASP from the skin biopsy samples for both groups. There were no differences

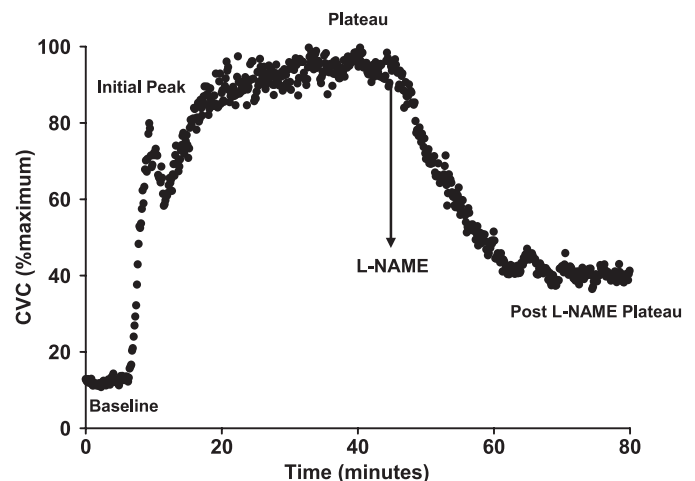


Fig. 1. A time course representative tracing of the control site during the local heating protocol. The baseline, initial peak, plateau, and post-L-NAME plateau are identified. CVC, cutaneous vascular conductance; L-NAME, *N*^G-nitro-L-arginine methyl ester.

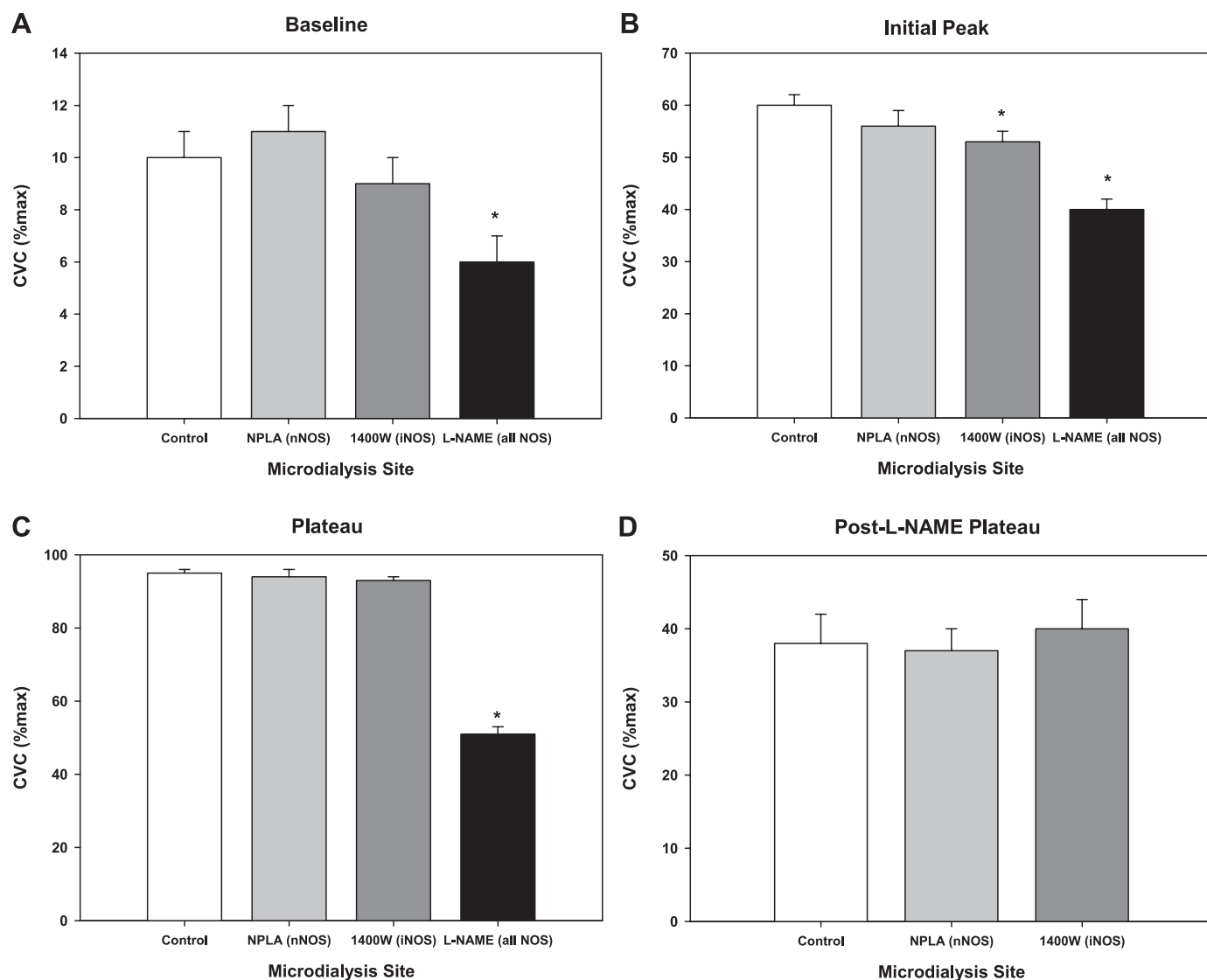


Fig. 2. Mean \pm SE percentage of maximal CVC (%CVC_{max}) in baseline (A), the initial peak (B), the plateau (C), and after nonspecific NO synthase (NOS) inhibition with L-NAME (D) in each microdialysis drug treatment sites. Inducible NOS (iNOS) inhibition with 1400W decreased the initial peak, and nonspecific NOS inhibition with L-NAME decreased baseline, the initial peak, and plateau during local heating. NPLA, *N*^ω-propyl-L-arginine; nNOS, neuronal NOS. **P* < 0.001 vs. control site.

in eNOS, nNOS, or iNOS expression between the groups. Further, there was no difference in the downstream vasodilatory target pVASP between groups.

DISCUSSION

The principal finding of the present study was that eNOS is the primary isoform mediating cutaneous NO-dependent vasodilation during local heating and perfusion of the endothelium-dependent agonist ACh. There was no effect of selective nNOS or iNOS inhibition compared with the control sites on the plateau response to local heating or during the ACh dose response, but there was a significant attenuation when all NOS isoforms were inhibited with L-NAME. Further, during local heating iNOS contributed to the sensory nerve-mediated initial peak suggesting an upregulation in iNOS activity associated with sensory nerve activation. A secondary finding of the present study was that deficits in functional NO-dependent

vasodilation can be detected in middle-aged skin. These deficits were only apparent during 1) local heating when NO-dependent vasodilation was quantified using a within-site perfusion of L-NAME and not from a comparison of the plateau %CVC_{max} values or 2) at the highest concentration of ACh (100 mmol/L). There were no differences in NOS isoform expression or downstream vasodilatory molecular targets (pVASP), suggesting the modest changes observed in function are likely due to changes in NO metabolism.

In young human skin there is controversy pertaining to the precise NOS isoform mediating the production of NO during the skin local heating response (18, 34). The use of different pharmacological inhibitors, patient populations, and regional skin circulations examined contributes to the inconsistent findings with these studies. The results from the present study suggest that eNOS is the primary NOS isoform mediating the production of NO during the plateau phase of the local heating

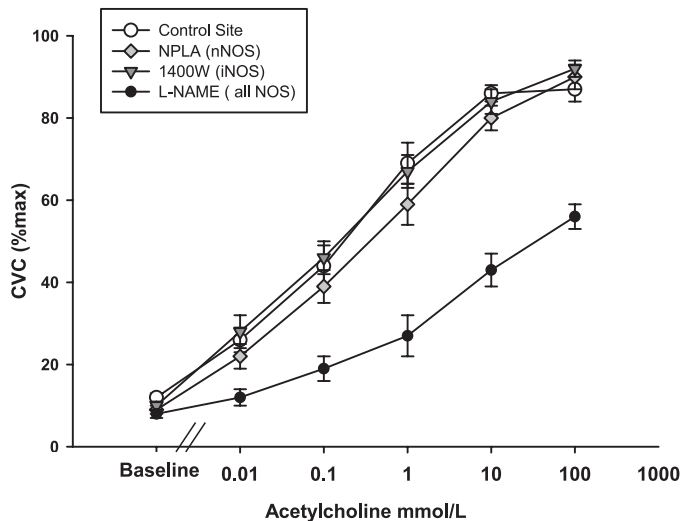


Fig. 3. Mean \pm SE %CVC_{max} during an ACh dose response. There was no effect of selective nNOS or iNOS inhibition. Nonspecific NOS inhibition with L-NAME attenuated %CVC_{max} at all concentrations of ACh. * $P < 0.001$, significant difference vs. control site.

response in young and middle-aged nonglaborous forearm skin. This is evidenced from sites treated with the specific nNOS and iNOS inhibitors having no effect on attenuating the plateau response and that nonspecific NOS inhibition with L-NAME at the plateau had a similar effect in all sites. It is possible that the concentration of the inhibitors used was not efficacious at blocking the specific NOS isozymes. However, we used the same concentration of NPLA used by Kellogg et al. (15), finding similar results during local heating. We also verified the efficacy of this inhibitor in pilot work using a whole body heating protocol and observed an attenuation in the NO-mediated portion of the reflex vasodilatory response. Furthermore, we used the same concentration of 1400W (iNOS

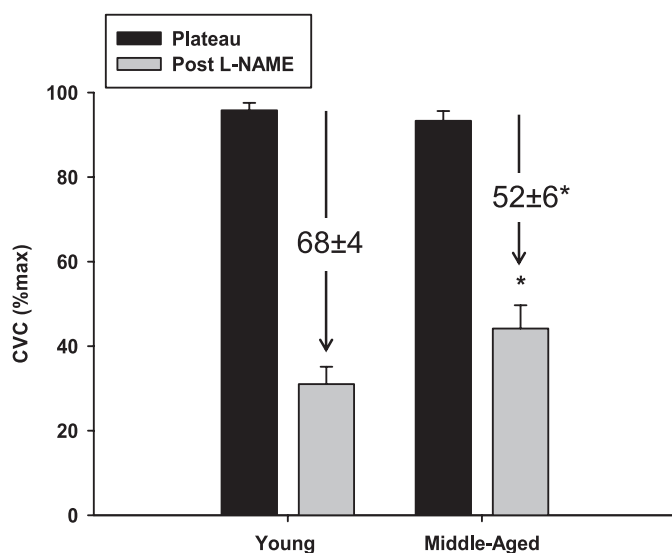


Fig. 4. Group mean \pm SE %CVC_{max} during the plateau and post-L-NAME plateau of the local heating response. The post-L-NAME plateau was increased and total NO-dependent vasodilation, illustrated as the difference between the plateau and the post-L-NAME plateau was decreased in the middle-aged group * $P = 0.013$.

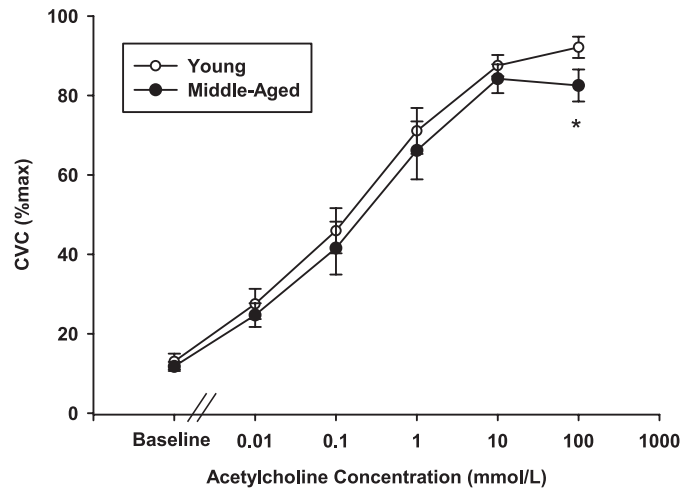


Fig. 5. Group mean \pm SE %CVC_{max} during an ACh dose response in the control sites. ACh-induced vasodilation was attenuated in the middle-aged at the two highest concentrations of ACh; * $P = 0.03$.

inhibitor) detecting significant effects specific to iNOS mechanisms in an essential hypertensive population (32). Together, these data confirm that the NO-mediated portion of the plateau response during local heating is likely produced via eNOS-dependent mechanisms.

One unexpected finding from the present study was that iNOS inhibition with 1400W reduced the initial sensory nerve-mediated peak in skin blood flow during local heating. Based on the putative role of iNOS as an inflammatory mediator that reduces eNOS-dependent mechanisms (30, 33) we did not anticipate that iNOS inhibition would attenuate the initial peak in healthy subjects. The initial peak is partially mediated by the production of NO, but there is a significant portion that is due to the release of neurogenic vasodilator peptides acting on neurokinin 1 receptors including the putative neurotransmitters substance P and CGRP (36). Moreover, it appears that adrenergic neurotransmitters also modulate this response (6). Our data suggest that iNOS-mediated NO production also contributes to the initial peak. Interestingly, in other tissues substance P has been shown to augment NO production through iNOS (13).

In the present study we utilized two skin-specific protocols to examine possible age-related changes in cutaneous microvascular function. These techniques are increasingly being utilized to examine mechanisms of microvascular dysfunction in clinical populations. Many of the disease states under investigation have an onset in middle-age and therefore clinical populations are often compared with a healthy middle-aged cohort. Furthermore, much of the work in the aging literature has compared a group of subjects of advanced age (65–85 yr) contrasted to a group of young healthy control subjects (18–30 yr) (26). In this study we have specifically examined a middle-aged group (46–57 yr). Our data indicate that deficits in cutaneous NO-mediated vasodilation can be detected in this middle-aged population. These deficits only become apparent when NO-dependent vasodilation is quantified within site by perfusing L-NAME during the plateau phase of the local heating response. Without this direct quantification there are no differences in the absolute plateau phase of the response which is in contrast to what we observe with advanced age or in

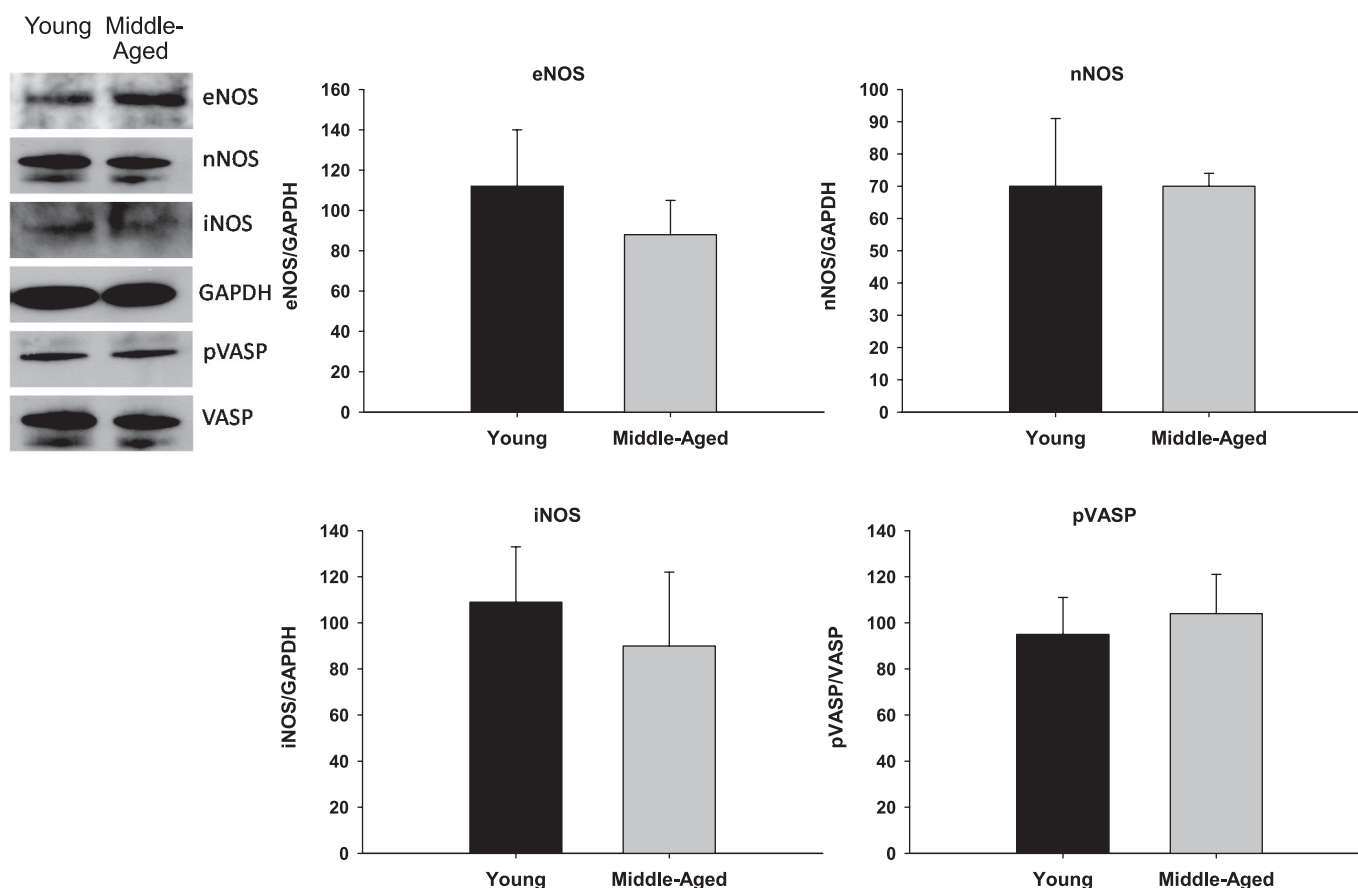


Fig. 6. iNOS, nNOS-2, endothelial NOS (eNOS)-3, VASP (vasodilator-stimulated phosphoprotein), and pVASP (phosphorylated VASP). Expression of the three NOS enzymes, and VASP, and pVASP activity were determined by Western blotting. GAPDH was used as loading control. Sample blot is shown in first panel. Densitometry analysis was performed using ImageJ software (NIH).

hypercholesterolemic (10) or essential hypertensive patients (32). These data suggest that a secondary NO-independent pathway is upregulated to compensate for the decrease in NO with healthy middle aging. In the context of the aging continuum this is an intriguing finding because it appears that the middle aged are able to compensate and that redundancy is lost in advanced age.

In middle-aged skin we also observed that endothelium-dependent vasodilation to the agonist ACh was reduced but only at the highest concentration tested (100 mmol/l). There was also no difference in the ACh-dose response in sites treated with the nonspecific NOS inhibitor L-NAME between age groups. In contrast, in an essential hypertensive population of the same age group where there is more clinically significant endothelial dysfunction we observe an augmented ACh-dose response in sites continuously treated with L-NAME. Taken together, these data show that modest age-related changes in cutaneous microvascular function specific to NO can be detected in healthy middle-aged skin.

In vitro analysis of skin biopsy samples showed that there was no difference in the expression of the NOS isoforms between age groups. Furthermore, there was no difference in the downstream vasodilatory target pVASP/VASP for Ser239, which is the major site at which the cGMP-dependent kinase PKG phosphorylates VASP. Functionally we detected modest declines in endothelium-dependent vasodilation in the middle-aged group. The biochem-

ical expression data do not show a significant difference between NOS isoform protein content or differences in pVASP between age groups, but these data are not reflective of mechanisms affecting NO such as increased oxidant stress. It is not surprising that we were unable to detect differences in pVASP as these proteins were measured in unstimulated whole skin homogenates and functionally only modest differences in eNOS-dependent vasodilation were measurable.

Limitations

Our aim with the present study was to examine the specific isoforms mediating cutaneous vasodilation during local heating. We further wanted to determine if there were any age-related changes to these mechanisms in middle-aged skin. We have previously examined similar mechanisms with primary advanced aging. In the present study it would have been ideal to have three age groups to determine the age-related changes along the aging continuum. While we are relying on historical data collected by our research group for the advanced age group, this does not detract from our ability to make conclusions about the mild cutaneous microvascular dysfunction that we measured in the middle-aged group. Moreover, this study enables us to put these middle-age-related changes in context with those changes that we observe in other clinical populations (9, 10, 26, 32).

We have utilized a standardized (8–10, 24, 26, 32) local heating protocol to induce NO-dependent vasodilation using the nonspecific NOS-inhibitor L-NAME to quantify vasodilation due to the production of NO during heating. To do this we continually heated the skin for 70–90 min. Hodges et al. (5) noted that with adrenergic blockade that heating for this length of time resulted in a “die-away phenomenon.” Because we did not include a time control it is possible that part of what we are attributing as vasodilation due to NO production may be accounted for with the die-away phenomenon. However, if this were the case the error would have been systematic across all sites and therefore does not change the main interpretation of the local heating data.

In addition to the functional data collected with isoform-specific inhibitors, we have analyzed skin samples from the same subjects to examine NOS isoform protein expression. Unfortunately, we were not able to measure NOS activity in these samples because of 1) limited protein recovery from the skin homogenates and because 2) there are many non-NOS-dependent endogenous sources of nitrate and nitrite in human skin resulting from ultraviolet light exposure (28). It would have also been ideal to obtain Western blot data of phosphorylated eNOS. This would have yielded greater insight into our results showing attenuated cutaneous vasodilation in middle-aged skin. However, because of the labile nature of phosphorylated proteins and the time lapse between sample analyses we were unable to make these measurements.

Finally, our conclusions are based off of establishing the efficacy of our isoform-specific inhibitors. There is evidence in knockout animal models and with prolonged isoform-specific inhibition that other isoforms may be upregulated. We cannot exclude this as a possibility, although we think it is unlikely because the relatively short time course of our isoform-specific inhibition would not be sufficient to observe an increase in protein expression. With regard to the specific isoform inhibitor efficacy we rigorously tested these inhibitors including performing additional whole body heating experiments to verify the results of Kellogg et al. (15) and relied on historical data from other laboratories to determine final inhibitor concentrations. We have also seen efficacious inhibition of iNOS with this concentration of 1400W in an essential hypertensive population, which was supported with molecular data (32). However, it is possible that isoform-specific NOS blockade was not fully achieved. In young subjects our data showed that at the intermediate concentrations of ACh that NPLA modestly inhibited vasodilation. Although we did see attenuation in NO-dependent vasodilation during reflex heating in our pilot work with young subjects and no effect on the local heating response in the present study, these data suggest to us that NPLA at this concentration may have also partially inhibited other NOS isoforms, likely eNOS.

Summary

In summary, our main finding was that eNOS is the primary isoform mediating cutaneous NO-dependent vasodilation during local heating and perfusion of the endothelium-dependent agonist ACh. Using these skin-specific techniques we found that modest deficits in functional NO-dependent vasodilation were present in middle-aged human skin. Additionally, NO derived from iNOS contributed to the sensory nerve-mediated

initial peak. Finally, from the skin biopsy analysis we observed no differences in NOS isoform expression or downstream vasodilatory molecular targets suggesting the modest changes observed in function are likely due to changes in enzyme activity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.S.B., L.S., A.E.S., C.J.S., and L.A.H. performed experiments; R.S.B., L.S., C.J.S., and L.A.H. analyzed data; R.S.B., L.S., D.E.B., W.L.K., and L.A.H. interpreted results of experiments; R.S.B., L.S., and L.A.H. prepared figures; R.S.B. and L.A.H. drafted manuscript; R.S.B., L.S., C.J.S., W.L.K., and L.A.H. edited and revised manuscript; R.S.B., L.S., A.E.S., C.J.S., D.E.B., W.L.K., and L.A.H. approved final version of manuscript; D.E.B. and L.A.H. conception and design of research.

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